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(54) Title: ESTROGEN RECEPTOR

(57) Abstract: Androgens regulate the male skeleton directly via a stimulation of androgen receptors and indirectly via aromatization of androgens into estrogen and thereafter stimulation of estrogen receptors (ER). In order to investigate the relative importance of estrogen receptor subtypes in the regulation of the male skeleton, the skeletal phenotypes of wild type (WT), ERα, Knockout (ERKO), ERβ Knockout (BERKO) and ERα/β Double Knockout (DERKO) mice were compared. ERKO and DERKO had reduced body weight as well as longitudinal bone growth. Furthermore, ERKO and DERKO but not BERKO demonstrated a pronounced decrease in bone mineral content in the long bones and in the axial skeleton. This decrease in BMC was due to cortical ostopenia as a result of decreased radial growth of the bones. Mechanical testing demonstrated that femora from ERKO were weaker as a result of the altered cortical bone dimensions. No significant change in trabecular BMD was seen in any group. ERKO demonstrated decreased serum levels of osteocalcin and IGF-I. Furthermore, serum levels of IGF-I were correlated to most of the skeletal changes seen in DERKO and ERKO. In conclusion , the skeletal phenotypes of DERKO and ERKO are similar and clearly distinguishable from WT and BERKO. Therefore, ERα, but not ERβ, mediates the effect of estrogen in the skeleton of male mice.

Estrogen Receptor

This invention relates to estrogen receptors and, particularly though not exclusively, to the effect of estrogen receptors and their ligands/modulators on the regulation of growth and bone-related parameters.

Several studies demonstrate that androgens are important in males. Orchidectomy decreases longitudinal growth and radial cortical growth in the long bones of rodents (Turner, R. T *et al* (1990) J. Orthop Res. **8**, 612-617; Turner, R. T *et al* (1989) J Bone Miner Res. **4**, 557-563; Sandstedt, J *et al* (1994) Endocrinology **135**, 2574-2580; Ornoy, A. *et al* (1994) Bone Miner **24**, 43-58). Furthermore, androgen treatment stimulates growth in orchidectomized growing rats and mice (Turner R. T. *et al* (1990) *supra*; Ornoy, A. *et al* (1994) *supra*; Jansson, J. O. *et al* (1985) Endocrinology **117**, 1881-1889) as well as in growing boys (Richman, R. A. & Kirschm, L. R. (1988) N Engl. J. Med. **319**, 1563-1567). These effects may either be direct via the stimulation of androgen receptors or indirect via aromatization of androgens into estrogen and thereafter stimulation of estrogen receptors. Recently it was demonstrated by Vanderschueren *et al* that the conversion of androgens into estrogen is required for normal body growth in male rats, indicating that indirect effects of androgens, mediated by estrogen, are important (Vanderschueren, D *et al* (1997) Endocrinology **138**, 2301-2307).

In addition to the growth-related effects of gonadal deficiency, orchidectomy also decreases bone mass in adult rodents (Turner R. T *et al* (1989) *supra*; Vanderschueren, D *et al* (1997) supra; Koh, E T *et al* (1996) Magnes Res. 9, 13-21). This effect is at least partly dependent on the androgen receptor as treatment with non-aromatizable androgens restores bone mass (Vanderschueren D *et al* (1992) *supra*; Wakley, G. K. *et al*) 1991) J Bone Miner Res. **6**, 325-330). On the other hand several clinical studies have demonstrated a strong relationship between serum estrogen levels and BMD in males (Slemenda, C. W. *et* al (1997) J Clin Invest 100, 1755-1759; Gillberg, P *et* al (1999) Calcif Tissue Int. 64, 209-213; Ongphiphadhanakul, B. *et* al (1995) Clin. Endocrinol (Oxf) 43, 727-733; Ongphiphadhanakul, B. *et al* (1998) Clin. Endocrinol (Oxf) **49**, 803-809). Furthermore,

aromatase deficiency in humans (Morishima, A *et al* (1995) J. Clin. Endocrinol. Metab. **80**, 3689-3698) as well as aromatase inhibition in rats (Vanderschueren, D. *et al* (1997) *supra*; Vanderschueren, D. *et al* (1996) Calcif Tissue Int. **59**, 179-183) is associated with osteopenia, suggesting that androgens may also regulate adult bone metabolism, either directly by stimulation of androgen receptors, or indirectly via aromatization and subsequent stimulation of estrogen receptors.

The cloning of the novel estrogen receptor, ERβ, suggested that there may exist alternative mechanisms of action for estrogen (Kuiper, G.G., *et al* (1996) *Proc. Natl. Acad. Sci. U S A* **93**, 5925-5930). We and others have demonstrated that ERβ is expressed in growth plate chondrocytes and osteoblasts, indicating a possible role for ERβ in the regulation of longitudinal bone growth and/or adult bone metabolism (Onoe, Y., *et al* (1997) *Endocrinology* **138**, 4509-4512, Arts, J., Kuiper, G.G., *et al* (1997) *Endocrinology* **138**, 5067-5070, Vidal, O., *et al* (1999) *J Bone Miner Res* In press, Nilsson, L.O., *et al* (1999) *J Clin Endocrinol Metab* **84**, 370-373; Windahl own unpublished results). However, the physiological role of ERβ in the regulation of growth and bone metabolism is still unknown. In humans, evidence for the importance of ERα for mediating effects of estrogen in the skeleton comes from a case report describing a young male with estrogen resistance due to a mutation in the human ERα gene (Smith, E. P. *et al* (1995) N. Engl. J. Med. **331**, 1056-1061). This male was reported to suffer from osteoporosis at the age of 28. Mice lacking a functional ERα gene, ERα Knockout mice (ERKO), have been generated (Couse, J. F. *et al* (1995) Mol. Endocrinol. **9**, 1441-1454) and more recently ERβ Knockout mice (BERKO) have also been described (Krege, J. H. *et al* (1998) Proc. Natl. Acad. Sci USA **95**, 15677-15682). At present the skeletal phenotype of male ERKO mice is unclear (Kimbro, K. *et* al (1996) J. Bone Miner Res. 11, S125; Schmidt, A. *et* al (1999) J Bone Miner Res. 14, S456; Ederveen. A. *et al* (1999) J Bone Miner Res. **14**, S170). Furthermore, we recently demonstrated that male BERKO mice do not exhibit osteopenia (Windahl, S. H. *et al* (1999) J Clin Invest. **104**, 895-901). This has raised the question concerning the relative importance of estrogen receptor subtypes in the skeleton of male mice. In order to investigate the estrogen receptor specificity in the regulation of growth and adult bone metabolism in male mice, we have generated Double-ER-Knockout mice

(DERKO). In the present study we have compared the skeletal phenotypes of male WT, ERKO, BERKO and DERKO mice.

We have recently generated mice devoid of functional ERβ protein and reported that ERβ is essential for normal ovulation efficiency, but is not essential for female or male sexual development, fertility, or lactation (Krege, J.H., *et al* (1998) *Proc Natl Acad Sci US A* **95**, 15677-15682).

The molecular mechanisms of action for ERα versus ERβ have recently been investigated. ERα and ERβ have almost identical DNA-binding domains and studies *in vitro* have demonstrated that the two receptors have similar affinities for estrogenic compounds (Kuiper, G.G. *et al* (1996) *Proc Natl Acad Sci U S A* **93**, 5925-5930, Kuiper, G.G., *et al* (1997) *Endocrinology* **138**, 863-870, Tremblay, G.B., *et al* (1997) *Mol Endocrinol* **11**, 353-365). The amino-acid sequence of ERβ differs from ERα in the N- and C-terminal trans-activating regions. Therefore the transcriptional activation mediated by ERβ may be distinct from that of ERα (Paech, K., *et al* (1997) *Science* **277**, 1508-1510). Considering the great similarities in ligand- and DNA- binding specificity it has been speculated that a differential tissue distribution of estrogen receptors may be important for mediating tissue specific responses to estrogens (Kuiper, G.G., and Gustafsson, J.A. (1997) *FEBS Lett* **410**, 87-90). Thus, the unique transactivating domains of the two receptor subtypes, in combination with differential tissue-distribution, or differential cell-type distribution within a tissue, could be important factors to determine the estrogen response in target tissues.

The hormone testosterone is required for the pubertal growth spurt and the acquisition of normal bone density in mammals. These effects of testosterone may be direct via stimulation of the androgen receptor, or indirect via aromatisation of testosterone and thereafter stimulation of estrogen receptors. In the present study, the inventors have looked at the role of estrogen receptor subtypes for pubertal growth and adult bone metabolism in male mammals, particularly male mice.

The effect of androgens on the male skeleton may either be direct via a stimulation of androgen receptors or indirect via aromatization of androgens into estrogen and thereafter stimulation of estrogen receptors. Possible direct effects of androgens are illustrated by

skeletal abnormalities in androgen resistant humans and rodents (Bertelloni. S. *et al* (1998) Horm. Res. **50**, 309-314, Vanderschueren, D. *et al* (1993) J. Bone. Miner. Res. 8, 801-809). However, several studies have clearly demonstrated that the effect of androgens on the male skeleton, at least partly, is dependent on the conversion of androgens into estrogen. In the present study, we demonstrate that estrogen resistance in the male mouse, due to loss of all known estrogen receptors, results in decreased skeletal growth. ERKO and DERKO but not BERKO mice display similar growth phenotypes, demonstrating that ERα but not ERβ is the estrogen receptor mediating the effects of estrogen on skeletal growth in the male mouse. The shortening of the long bones in ERKO and DERKO mice was associated with decreased growth plate width in the proximal tibia. Similar findings have also been reported in orchidectomized mice and rats (Turner, R. T. *et al* (1989) *supra*; Sandstedt, J. *et al* (1994) *supra*). Furthermore, Ornoy *et al* showed that orchidectomy in mice decreases growth plate area measured in the proximal tibia and that low-dose estrogen treatment increases the same parameter (Ornoy, A *et al* (1994) *supra*). These findings demonstrate that physiological levels of estrogen have a stimulatory effect on longitudinal growth in male rodents. Similarly, estrogens are required for the pubertal growth spurt in boys (MacGillivray, M. H. *et al* (1998) Horm. Res. **49 Suppl** 1, 2-8). Estrogen regulates final height in humans by a stimulatory effect on the pubertal growth spurt, followed by closure of the epiphyseal growth plates at the end of puberty. In humans with estrogen deficiency or estrogen resistance growth plate fusion never occurs. This results in continuous slow growth even after puberty (Morishima, A. *et al* (1995) J. Clin. Endocrinol. Metab. **80**, 3689-3698, Smith, E. P *et al* (1994) N. Engl. J. Med **331**, 1056-1061). In rodents, on the other hand, growth plate closure does not occur. Therefore rodent species grow continuously throughout life. Thus, the decreased growth observed in ERKO and DERKO is caused by a lack of estrogen stimulated growth, whereas the tall stature in the previously described estrogen resistant adult male was caused by a lack of growth plate closure (Smith, E. P. *et al* (1994) *supra*). Therefore, the male ERKO mouse is not a good model for postpubertal growth in humans but it may be a model for skeletal growth during adolescence.

It is a well-established fact that orchidectomized rodents as well as hypogonadal humans develop osteopenia (Vanderschueren, D (1996) Horm. Res. **46**, 95-98; Seeman, E *et* al

(1983) Am. J. Med. **75** 977-983; Stanley, H. L. *et al* (1991) J Am. Geriatr. Soc. **39** 766-771). Although androgen replacement restores bone mass in gonadectomized male rats (Wakley, G. K. *et al* (1991) *supra*) it has also been demonstrated that estrogen, at least partly, reverses bone loss caused by orchidectomy (Ornoy. A. *et al* (1994) *supra*; Vanderschueren, D. *et al* (1992) *supra*). The present study, with estrogen insensitivity due to inactivation of both ERα and ERβ, supports the notion that estrogen exerts important effects on the male skeleton. The phenotype of the male DERKO mouse is similar to what has earlier been described for aromatase inhibited male rats (Vanderschueren, D. *et al* (1997) *supra*). Both male DERKO mice and aromatase inhibited male rats demonstrate decreased femoral BMC, areal BMD, as well as decreased cortical dimensions and moment of inertia, without any effect on cortical volumetric BMD or cortical thickness. These two studies demonstrate that the conversion of androgens into estrogen is important in male rodents and that skeletal maturation in such species is estrogen dependent. In the present study, BMC measured by DXA and adjusted for body weight, was significantly reduced in ERKO and DERKO males, demonstrating that the effects on BMC were specific and not only reflected a general growth inhibition. This finding clearly demonstrates that ERα alone, and not ERβ, is the mediator of estrogenic effects in the skeleton of mammals such as the male mouse. Interestingly, a slight decrease in the relative weights of the heart and lung was also seen in ERKO but not in BERKO mice, indicating that ERα may exert specific effects in these two organs as well. In contrast, the relative weights of liver, kidney and brain were unchanged in ERKO, BERKO and DERKO males.

ERKO and DERKO mice demonstrated a decreased diaphyseal cross sectional area and periosteal circumference of femur, resulting in a pronounced decrease of the area moment of inertia. When the quality of the bone is unchanged, the area moment of inertia is normally proportional to the mechanical strength of the bone determined by three-point-bending (Ferretti, J. L. *et al* (1996) Bone **18**, 97-102). The maximal load was decreased in male ERKO mice but it was not decreased more than suggested by the changes in area moment of inertia. Therefore, the amount of bone, but not the mechanical quality of the bone, was decreased in ERα inactivated male mice.

Aromatase inhibition of male rats resulted in a small decrease in trabecular BMD (Vanderschueren, D. *et al* (1997) *supra*). In the present study, neither the pQCT technique nor bone histomorphometry detected any significant changes in cancellous bone density in male ERKO, BERKO or DERKO mice. Thus, our experiments indicate that neither ERα nor ERβ is essential for the maintenance of cancellous bone mass in the male mouse. This finding raises the question whether other estrogen receptor subtypes exist or whether other hormones may compensate for estrogen resistance in the skeleton of male DERKO mice. Androgens prevent cancellous osteopenia in orchidectomized rats. Therefore androgens could compensate for loss of estrogen receptor activity in ERKO, BERKO and DERKO males. Interestingly, ERKO males have somewhat increased serum levels of testosterone (Eddy, E. M *et al* (1996) Endocrinology. **137**, 4796-4805).

Bone loss following gonadal deficiency is normally associated with increased bone turnover. Surprisingly, osteocalcin, a marker for bone formation, was decreased in ERKO males. This finding and the pronounced cortical osteopenia seen in ERKO and DERKO males led us to seek other explanations to the skeletal phenotype in these mice. Over-all size and cortical radial growth are parameters that are highly sensitive to changes in the GH/IGF-I axis (Andreassen, T. T. (1995) J. Bone. Miner Res. **10** 1057-1067, Ohlsson, C. *et* al (1998) Endocr. Rev. **19** 55-79; Rosen, H. N. *et al* (1995) J. Bone. Miner. Res. **10**, 1352-1358). Because these parameters were altered in ERKO and DERKO males, serum IGF-I was measured to investigate if the GH/IGF-I axis was affected in ERKO and DERKO males. Serum IGF-I levels were decreased in ERα inactivated mice. We also found a strong correlation between serum IGF-I levels and affected skeletal parameters in the ERKO and DERKO mice, including length, BMC of femur, periosteal circumference and maximal load in the femur diaphysis. These findings do not prove, but indicate, that changes in the GH/IGF-I axis could partly explain the skeletal phenotype seen in male ERKO and DERKO mice. GH and IGF-I are known to increase serum osteocalcin (Ohlsson. C. *et al* (1998) Endocr. Rev. **19**, 55-79). Therefore, the decreased serum osteocalcin levels in male ERKO mice may be caused by reduced serum IGF-I levels. This is also supported by the finding that aromatase inhibited male rats have decreased serum IGF-I levels and reduced levels of serum osteocalcin (Vanderschueren, D. *et al* (1997) *supra*). An effect of estrogen on the GH/IGF-I axis in males is also supported by several

clinical as well as experimental studies. Circulating GH and IGF-I concentrations increase during normal male puberty (Miller, J. D. *et* al (1982) J. Clin. Endocrinol Metab. **55** , 989-994; Mauras, N. *et* al (1987) J. Clin Endocrinol Metab **64** 596-601; Martha Jr. P M. *et al* (1989) J. Clin. Endocrinol. Metab **69**, 563-570; Weissberger. A. J. *et al* (1989) Horm Res. **32**, 148-150). These changes appear to be secondary to the pubertal rise in testosterone concentrations since they are also observed in prepubertal and hypogonadal boys undergoing induction of puberty with exogenous testosterone (Miller, J. D. *et al* (1982) *supra*; Link, K. *et al* (1986) J. Clin. Endocrinol Metab. **62**, 159-164). The mechanism whereby testosterone interacts with the somatotropic axis may either be direct, mediated by androgen receptors, or indirect through the action of estrogen on estrogen receptors. The possibility that estrogen mediates the effects of testosterone on the somatotropic axis has been suggested in a previous study showing a significant correlation between circulating levels of estrogen, but not testosterone, and GH secretion in men (Ho. K. Y. *et al* (1987) J. Clin. Endocrinol Metab. **64**, 51-58). Furthermore, it has also been demonstrated that testosterone plays an important role in the modulation of the somatotropic axis in adulthood and this effect is, at least partly, dependent on the conversion of testosterone to estrogen (Weissberger, A. J. *et al* (1993) J. Clin. Endocrinol. Metab **76**, 1407-1412).

The effects of androgens in the skeleton of the male mouse are summarised in Fig. 8. Others have presented studies indicating that androgens, directly via interaction with the androgen receptor, exert effects on the male skeleton. In the present study we have confirmed that part of the effect of androgens is dependent on aromatization. Furthermore, the present study clearly demonstrates that ERα, but not ERβ, mediates the effect of estrogen on the skeleton in the male mouse. In conclusion, we have generated DERKO mice, which are fully viable despite the fact that they are devoid of all known estrogen receptors. Male ERKO and DERKO mice have decreased body weight, reduced longitudinal bone growth and a pronounced cortical osteopenia. Our findings demonstrate that ERα but not ERβ mediates the effect of estrogen in the male skeleton. We propose that some of the skeletal effects seen in ERα inactivated male mice may be due to an inhibition of the GH/IGF-I axis.

According to one aspect of the invention, there is provided a method of treating growth disorders in a mammal, the method comprising treating the mammal with an ERα-specific agonist.

According to another aspect of the invention, there is provided a method of treating growth disorders in a mammal, the method comprising treating the mammal with an ERα-specific antagonist.

The ERα ligand/modulator used in the method of the invention may be a SERM (Selective Estrogen Receptor Modulator) i.e a compound having a tissue-selective mixed agonist/antagonist activity. SERMs include tamoxifen, raloxifene, drolixifene and tamoxifen methiodide.

The mammal may be male or female and is preferably pre-pubesent.

The ERα agonist or antagonist used in the method may have a binding affinity of less than 10nM for ERα.. Preferably, the ERα agonist or antagonist has a binding affinity of 0.0001 to 10 nM for ERα.

According to another aspect of the invention, there is provided the use of an ERα selective agonist in the preparation of a medicament for the treatment of a growth disorder.

According to another aspect of the invention, there is provided the use of an ERα selective antagonist in the preparation of a medicament for the treatment of a growth disorder.

In such uses the ERα antagonist may have a binding affinity of ERα of less than 10 nM, preferably 0.0001 to 10 nM.

According to another aspect of the invention, there is provided a pharmaceutical composition suitable for treating or preventing growth disorders in a mammal, the composition comprising an ERα antagonist or agonist. Preferably, the ERα agonist or

antagonist has a binding affinity for ERα of less than 10 nM, most preferably 0.0001 to 10 nM.

Pharmaceutical compositions of this invention comprise any of the compounds of the present invention, and pharmaceutically acceptable salts thereof, with any pharmaceutically acceptable carrier, adjuvant or vehicle. Pharmaceutically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulphate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

The pharmaceutical compositions of this invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. The pharmaceutical compositions of this invention may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or vehicles. The term parenteral as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intra-articular, intrasynovial, intrasternal, intrasternal, intrathecal, intralesional and intracranial injection or infusion techniques.

The pharmaceutical compositions may be in the form of a sterile injectable preparation, for example, as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as, for example, Tween 80) and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are

conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or caster oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant such as Ph. Helv or a similar alcohol.

The pharmaceutical compositions of this invention may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, and aqueous suspensions and solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are administered orally, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring and/or colouring agents may be added.

As the skilled artisan will appreciate, lower or higher doses than those recited above may be required. Specific dosage and treatment regiments for any particular patient will depend upon a variety of factors, including the activity of the specific compound employed, the age, body weight, general health status, sex, diet, time of administration, rate of excretion, drug combination, the severity and course of the disease, the patient's disposition to the disease and the judgment of the treating physician.

According to another aspect of the invention, there is provided a method of selecting compounds for the regulation of body growth in mammals, the method comprising selecting a compound on the basis of its ability to antagonise agonist-dependent ERα activity.

According to a further aspect of the invention, there is provided a method of selecting compounds for the use in the treatment of growth disorders, the method comprising testing the compound in a mammal which is wholly or partially ERα deficient or in cells derived from such an animal.

According to the invention, there is also provided a method of treating a bone mineral density disorder in a mammal, the method comprising treating the mammal with an ERα -specifc agonist. Alternatively, the invention provides a method of treating a bone mineral density disorder in a mammal, the method comprising treating the mammal with an ERα -specific antagonist. The ERα -specific ligand/modulator may be a SERM, the mammal may be a male or female and may be pre-pubesent. The ERα agonist or antagonist may have a binding affinity of less than 10 nM, preferably 0.0001 to 10 nM or ERα.

The invention also provides the use of an ERα selective agonist in the preparation of a medicament for the treatment of a bone mineral density disorder.

Alternatively the invention provides the use of an ERα selective antagonist in the preparation of a medicament for the treatment of a bone mineral density disorder.

The invention also provides a pharmaceutical composition suitable for treating or preventing bone mineral density disorders in a mammal, the composition comprising an ERα antagonist or agonist.

The invention also provides a method of selecting compounds for the regulation of bone mineral density in mammals, the method comprising selecting a compound on the basis of its ability to antagonise agonist-dependent ERα activity. In particular, compounds are selected for the regulation of adult bone mineral density disorders.

Other aspects of the invention are apparent from the claims.

Methods in accordance with the invention will now be described, by way of example only, with reference to the accompanying drawings, Figures 1 to 8 in which:

Fig 1 shows the results of experiments on weight gain in male mice;

Fig, 2 illustrates the body weight in wild type (WT), ERKO, BERKP and DERKO mice at different ages.

Fig. 3 illustrates the results of experiments of Organ weights/Body weight expressed as % of wild type mice (WT) at 4 months of age in wild type (WT), ERKO, BERKO and DERKO (n=6 for WT, n=9 for ERKO, n=5 for BERKO and n=5 for DERKO).

Fig. 4 illustrates the results of experiments of Length of femur (A) and crown rump (B) and width of the proximal tibial growth plate (C) in wild type (WT), ERKO, BERKO and DERKO mice (n=6 for WT, n=9 for ERKO, n=6 for BERKO and n=5 for DERKO).

Fig. 5 shows the results of tests on bone mineral density in rats.

Fig 6 illustrates the results of experiments of DXA measurements of bone parameters in wild type (WT), ERKO, BERKO and DERKO mice (n=6 for WT, n=9 for ERKO, n=6 for BERKO and n=5 for DERKO);

Fig. 7 are representative DXA scans (A) and mid-diaphyseal pQCT scans (B) of femora in adult wild type (WT), ERKO, BERKO and DERKO mice. (bottom 4A: High = high bone mineral density and Low = low bone mineral density);

Fig. 8 illustrates the effects of androgens in the male mouse skeleton. AR = androgen receptor, ERα = estrogen receptor-α, ERβ = estrogen receptor-β.

1. Generation of Knockout Mice

Male double heterozygous ($ER\alpha^{+/-}$ $\beta^{+/-}$) mice were mated with female double heterozygous ($ER\alpha^{+/-}$ $\beta^{+/-}$) mice resulting WT, ERKO, BERKO and DERKO offspring. All mice were of mixed C57BL/65/129 backgrounds.

The animals were maintained under standardised environmental conditions, with free access to food and water. Genotyping of tail DNA was performed at 3 weeks of age. The *ERα*-gene was analysed with the following primer pairs: Primers

AACTCGCCGGCTGCCACTTACCAT and CATCAGCGGGCTAGGCGACACG for the WT gene, correspond to flanking regions in the targeted exon no. 2. They produce a fragment of approximately 320 bp. Primers TGTGGCCGGCTGGGTGTG and GGCGCTGGGCTCGTTCTC for the KO gene, correspond to part of the NEO-cassette and the flanking exon no. 2. They produce a 700 bp fragment. Genotyping of the *ERβ*-gene has been previously described (30).

2. Body Growth

Male wild type (WT) as well as estrogen receptor β -/- (BERKO) mice demonstrated a pubertal growth spurt as measured with body weight gain/day (Fig. 1) In contrast, no pubertal growth spurt was seen in estrogen receptor α -/- mice (ERKO) or in mice devoid of both estrogen receptors (DERKO).

In the results shown in Fig. 2, values are given as means. The bodyweights in WT and ERKO mice at different ages were first analysed by a two-way analysis of variance followed by Student Newman Keuls multiple range test. P values versus WT mice are indicated. Body weight was unchanged in ERKO, BERKO and DERKO at the prepubertal stage when compared to WT littermates (Fig 2, day 17, one-way ANOVA). Late pubertal and adult weight was decreased in ERKO and DERKO but not in BERKO when compared to WT mice (Fig 2, day 46-81, two-way ANOVA).

Growth of the appendicular- as well as the axial- skeleton was followed using repeated X-ray measurements. In the results shown in Fig. 3, values are given as means ± SEM. Data at different ages were first analysed by a two-way analysis of vairance (A and B) or by a one-way analysis of variance (C) followed by Student Newman Keuls multiple range test. P values versus WT mice are indicated in A and B. **$p<0.05$ versus WT (C). The length of the femur was chosen as a measure of appendicular growth whereas crown-rump (CR) length was used as a measure of axial growth. The length of the femur was unchanged at the prepubertal stage (Fig 3A, day 31, one-way ANOVA). Thereafter ERKO and DERKO demonstrated a gradual decrease in growth rate, resulting in a decreased femoral length at the adult stage (ERKO –5.7%, DERKO –4.4% versus WT, Fig 3A, 5A). The decreased

growth of the long bones in ERKO and DERKO was associated with a decreased growth plate width measured in the proximal tibia (Fig 3C). The CR length was also decreased in ERKO and DERKO compared with WT (Fig 3B).

3. Dual X-Ray Absorptiometry (DXA)

Areal Bone mineral density (Areal BMD; BMC/cm^2) and bone mineral content (BMC) were measured with the Norland pDEXA Sabre (Fort Atkinson, WI) and the Sabre Research software (3.6) as previously described (30).

In vivo measurements of animals were performed in order to determine total body, spine, femur and cranium BMC (medium resolution scan with line spacing set at 0.05 cm). Three mice were analysed at a time. A mouse, which was sacrificed at the beginning of the experiment, was included in all the scans as an internal standard in order to avoid inter-scan variations.

Ex vivo measurements of the left femur and tibiae were performed on excised bones placed on a 1 cm thick plexiglass table. All bones compared were measured in the same scan (high-resolution scan with line spacing set at 0.01 cm).

4. Peripheral Quantitative Computerized Tomography (pQCT)

Computerized tomography was performed with the Stratec pQCT XCT Research M (Norland, software version 5.4B) operating at a resolution of 70 μm as previously described (30).

Mid-diaphyseal pQCT scans of femora and tibiae were performed to determine the cortical volumetric bone mineral density (volumetric BMD), cortical cross sectional area, periosteal and endosteal circumference and the cross sectional moment of inertia. The mid-diaphyseal region of femora and tibiae in mice contains only cortical bone.

Metaphyseal pQCT scans of left femora and tibiae were performed to measure trabecular volumetric BMD. The scan was positioned in the metaphysis at a distance from the distal growth plate corresponding to 4 % of the total length of the femur (an area containing cortical as well as trabecular bone). The trabecular bone region was defined by setting an

inner threshold to 45% of the total area. The inter-assay coefficients of variation (CV) for the pQCT measurements were less than 2%.

The DXA technique gives the areal BMD whereas the pQCT gives the true volumetric BMD. Therefore a factor regulating the outer dimensions of a bone, will affect the areal BMD (DXA) but not the volumetric BMD (pQCT).

5. Histological examination and Histomorphometry

Growth plate measurements: Right and left tibiae were fixed in 4% formaldehyde, embedded in paraffin and sectioned at a thickness of 4 μm. The width of the growth plate was measured, after staining with Alcian blue/Van Gieson, using an image-processing system (Easy Image, Bergströms Instrument, Stockholm, Sweden) coupled to a microscope. The average of 20 growth plate measurements (2 sections, 10 measurements/section) was calculated for each tibia.

Bone Histomorphometry: The areas of trabecular bone within a reference area of the proximal tibia were measured in sections stained with Hematoxylin/Eosin. Measurements were performed on printed copies by point counting using a square lattice (1 and 2 cm). Three fields of vision on three sections from each animal were used for the analysis. Data is presented as the ratio of trabecular bone volume (BV) to total volume (TV).

6. Radioimmunoassay

Serum IGF-1 levels were measured by double antibody IGF binding protein-blocked radio immunoassay according to Blum and Breier (31).

7. Statistical Procedure

Dynamic measurements were first analysed by a two-way analysis of variance (ANOVA) followed by Student Newman Keuls multiple range test. Static measurements (at the time

of sacrifice) were first analysed by one-way ANOVA followed by Student Newman Keuls multiple range test.

8. Bone mineral status as determined by DXA

BMC (g) and areal BMD (mg/mm^2) were measured with DXA. In the results depicted in Fig. 5, the BMC (A) and BMC/Body weight (B) of the whole skeleton (total), femur, spine and cranium were measured using DXA technique as described in Methods. Values are given as means ± SEM. Data at different ages were first analysed by a two-way analysis of variance followed by Student Newman Keuls multiple range test. P values versus WT mice are indicated. BERKO demonstrated unchanged BMC and areal BMD (Fig 5A, table 1). Furthermore, in ERKO and DERKO no effect was seen on BMC and areal BMD at the prepubertal stage (day 31, one-way ANOVA). However, later on ERKO and DERKO presented a marked decrease in total body BMC (Fig 5A). In addition regional measurements of BMC in the femur and spine also showed a significant decrease (day 118; total body: ERKO –21%, DERKO –22%; femur: ERKO –23%, DERKO –20%; spine: ERKO –23%, DERKO –19%, versus WT; Fig 5A, 6A). In the results in Fig. 6A, High = high bone mineral density and Low = low bone mineral density. Only a small effect was seen in the cranium (ERKO –7% versus WT, Fig 5A). Total body areal BMD was slightly decreased in ERKO at the adult stage. Both ERKO and DERKO displayed a decreased adult areal BMD in the femur (Table 1).

Table 1. Areal BMD as Measured using DXA

		WT (n=6)	ERKO (n=9)	BERKO (n=6)	DERKO (n=5)
Total Body BMD (mg/cm^2)	Day 31	48.7±0.8	47.2±0.3	48.4±0.7	50.6±0.5
	Day 65	59.0±0.7	58.2±0.6	59.5±0.6	58.6±0.2
	Day 118	66.5±0.2	65.2±0.7	66.4±0.3	65.8±0.8
	2-way ANOVA		P<0.05	NS	NS
Femur BMD (mg/cm^2)	Day 31	35.1±0.9	33.3±0.5	34.5±0.7	35.5±1.2
	Day 65	58.2±1.3	52.9±1.8	55.0±1.7	51.0±2.6

	Day 118	64.3±1.6	58.9±1.3	64.0±1.9	60.8±2.1
	2-way ANOVA		P<0.01	NS	P<0.05
Spine BMD (mg/cm²)	Day 31	36.6±0.9	36.1±0.6	35.7±1.1	37.2±0.7
	Day 65	53.0±0.8	51.5±0.8	53.4±1.0	49.5±1.9
	Day 118	61.2±0.8	56.8±1.1	61.2±1.5	60.8±3.0
	2-way ANOVA		NS	NS	NS

Values are given as means ± SEM Data at different ages were first analysed by a two-way analysis of variance followed by Student Newman Keuls multiple range test. P values versus WT mice are indicated. NS = non significant.

To determine if the decrease in BMC in ERKO and DERKO males was greater than that associated with retarded body growth, BMC/body weight was calculated for the whole skeleton and for individual bones. Interestingly, in adult mice total body BMC/body weight was decreased in ERKO (-18%) and DERKO (–22%) when compared to WT. This was also the case for femur (ERKO –20; DERKO –19%) and spine (ERKO -21%; DERKO –18%; Fig 5B).

9. Cancellous [what does this term mean?] bone density

The pQCT technique was used to measure trabecular volumetric BMD in the metaphysis of the distal femur and in the proximal tibia. Results are shown in Table 2

Table 2. Trabecular volumetric BMD and Cortical Bone Parameters of Femur as Measured using pQCT

	WT (n=6)	ERKO (n=9)	BERKO (n=6)	DERKO (n=5)
Trabecular density (mg/mm³)	0.312±0.021	0.293±0,011	0.268±0.021	0.285±0.019
Cortical density (mg/mm³)	1.188±0.016	1.189±0.006	1.193±0.011	1.184±0.011
Cortical area (mm²)	1.06±0.02	0.91±0.02**	1.01±0.04	0.92±0.04*
Cortical bone mineral content (mg/mm)	1.26±0.04	1.08±0.03*	1.21±0.06	1.09±0.05*
Cortical periosteal circumference (mm)	5.65±0.08	5.15±0.06**	5.57±0.11	5.26±0.12*
Cortical endosteal circumference (mm)	4.31±0.13	3.89±0.05*	4.28±0.09	4.02±0.11

Values are given as means ± SEM. Data were first analysed by a one-way analysis variance followed by followed by Student Newman Keuls multiple range test. * $p< 0.05$. ** $p< 0.01$ versus WT.

In addition, histomorphometry was performed in the metaphysis of the proximal tibia, where trabecular bone volume/total volume (BV/TV) was measured. Neither the pQCT technique (Table 2, and data not shown) nor bone histomorphometry (BV/TV: WT 0.32±0.05; ERKO 0.32±0.02; BERKO 0.33±0.02; DERKO 0.34±0.02; one-way ANOVA) detected any significant changes in cancellous bone density.

10 Cortical bone parameters

Cortical bone parameters were studied in detail in mid-diaphyseal pQCT scans of femora and tibiae (Table 2, Fig 6B and data not shown). The cortical BMC in the mid-diaphyseal section of femur was decreased in ERKO (-14%) and DERKO (-14%) compared with WT and this decrease was mainly due to a decreased cross-sectional bone area whereas cortical volumetric density was unchanged (Table 2). The decrease in cross sectional area in ERKO and DERKO was associated with decreased periosteal and endosteal circumference (Fig 6B and Table 2).

11 Mechanical testing of the femur diaphysis

The size and position of the cortical cross-sectional bone area in ERKO and DERKO resulted in a pronounced decrease of cortical cross-sectional moment of inertia (ERKO –29%, DERKO –24% versus WT, Table 3).

Table 3. Mechanical testing of Femur Diaphysis

	WT (n=6)	ERKO (n=9)	BERKO (n=5)	DERKO (n=5)
Area Moment of Inertia (mm^4)	0.34±0.01	0.24±0.01**	0.32±0.03	0.26±0.02*
Maximal Load (N)	28.4±1.9	23.2±0.7*	26.5±2.1	24.3±1.6
Elastic Modulus (MPa)	3.8±0.2	4.4±0.3	3.5±0.3	3.5±0.1
Maximal Stress (GPa)	132±8	140±5	122±10	140±6

Values are given as means ± SEM. Data were first analysed by a one-way analysis variance followed by followed by Student Newman Keuls multiple range test. * $p< 0.05$. * $p< 0.01$ versus WT.

Changes in area moment of inertia are often directly correlated to changes in the mechanical strength of the bone. Therefore, mechanical strength was tested by three-point-bending at the mid-diaphysel region of femur. ERKO demonstrated a significantly decreased maximal load whereas a tendency to decrease was seen in DERKO (ERKO –18%, DERKO –15%) compared with WT (Table 3). Other bone parameters, including maximal stress and elastic modulus, reflecting the quality of the bone, were not statistically changed (Table 3).

12. Biochemical Bone markers and IGF-I in serum

Osteocalcin, a marker of bone formation was measured in serum at 110 days of age. Osteocalcin was decreased in ERKO (Osteocalcin –25%, Table 4) and a tendency to decrease was seen in DERKO (Osteocalcin –9%, Table 4).

Table 4. Biochemical Bone Markers and IGF-I in Serum

	WT (n=6)	ERKO (n=9)	BERKO (n=6)	DERKO (n=5)
Osteocalcin (ng/ml)	94±3	71±3*	93±6	86±11
IGF-I (ng/ml)	337±36	250±8*	313±12	264±6

Values are given as means ± SEM. Data were first analysed by a one-way analysis variance followed by followed by Student Newman Keuls multiple range test. * $p< 0.05$. ** $p< 0.01$ versus WT.

Overall size and cortical radial growth are parameters, which are highly sensitive to changes in the GH/IGF-I axis. Because these parameters were altered in ERKO and DERKO males serum IGF-I levels were measured to investigate if the GH/IGF-I axis was affected in the ERKO and DERKO. Serum IGF-I levels were decreased in ERKO (-26%)

and there was a tendency to a decrease in DERKO (-22%, Table 4). Serum IGF-I levels were statistically correlated with length, BMC, BMC/weight, cortical cross sectional area, periosteal circumference, moment of inertia and ultimate load of femur (Table 5).

Table 5. Correlation with serum IGF-I

		r
Femur	Length	0.60**
	BMC	0.70***
	BMC/weight	0.59**
	Trab vol BMD	0.22
	Cortical cross sectional area	0.55**
	Endosteal circumference	0.47*
	Periosteal circumference	0.54**
	Moment of Inertia	0.55**
	Ultimate Load	0.50*
Liver	Weight	0.18
Kidney	Weight	0.04
Heart	Weight	0.39
Lung	Weight	0.07
Brain	Weight	-0.17

Correlations were calculated using Pearsons correlation coefficient (r). * $p<0.05$, ** $p< 0.01$, *** $p<0.001$.

13. Organ weights

The weights of several other organs were measured to see if the effect on the skeleton in ERKO and DERKO was tissue specific. To compare the relative growth of different organs the individual organ weights were divided with the total body weight. The weights of the liver, kidney, brain and testis were not significantly changed in any group. However, the weights of heart and lung were decreased in the ERKO compared with WT (heart –15%, lung –17%, Fig 7). In the results shown in Fig. 7, values are given as means ± SEM. Data were first analysed by a one-way analysis of variance followed by Student Newman Keuls multiple range test. * $p<0.05$ versus WT.

These experiments demonstrate that ERα but not ERβ is involved in the regulation of pubertal growth and adult bone mineral density in male mammals such as mice.

Claims

1. A method of treating growth disorders in a mammal, the method comprising treating the mammal with an ERα-specific agonist.

2. A method of treating growth disorders in a mammal, the method comprising treating the mammal with an ERα-specific antagonist.

3. A method according to claim 1 or 2, in which the mammal is male or female.

4. A method according to claim 1 or 2, in which the mammal is pre-pubesent.

5. A method according to any preceding claim, in which the ERα agonist or antagonist has a binding affinity of less than 10nM or ERα.

6. A method according to claim 5, in which the ERα agonist or antagonist has a binding affinity of 0.0001 to 10 nM for ERα.

7. The use of an ERα selective agonist in the preparation of a medicament for the treatment of a growth disorder.

8. The use of an ERα selective antagonist in the preparation of a medicament for the treatment of a growth disorder.

9. The use according to claim 8 in which the ERα antagonist has a binding affinity of ERα of less than 10 nM.

10. The use according to claim 9, in which the ERα antagonist has a binding affinity of 0.0001 to 10 nM to ERα.

11. A pharmaceutical composition suitable for treating or preventing growth disorders in a mammal, the composition comprising an ERα antagonist or agonist.

12. A pharmaceutical composition according to claim 11, in which the ERα agonist or antagonist has a binding affinity for ERα of less than 10 nM.

13. A pharmaceutical composition according to claim 12, in which the ERα agonist or antagonist has a binding affinity for ERα of 0.0001 to 10 nM.

14. A method of selecting compounds for the regulation of body growth in mammals, the method comprising selecting a compound on the basis of its ability to antagonise agonist-dependent ERα activity.

15. A method of treating a bone mineral density disorder in a mammal, the method comprising treating the mammal with an ERα -specific agonist.

16. A method of treating a bone mineral density disorder in a mammal, the method comprising treating the mammal with an ERα -specific antagonist.

17. A method according to claim 15 or 16, in which the mammal is male or female.

18. A method according to claim 15 or 16, in which the mammal is pre-pubesent.

19. A method according to any preceding claim, in which the ERα agonist or antagonist has a binding affinity of less than 10nM for ERα.

20. A method according to claim 19, in which the ERα agonist or antagonist has a binding affinity of 0.0001 to 10 nM for ERα.

21. The use of an ERα selective agonist in the preparation of medicament for the treatment of a bone mineral density disorder.

22. The use of an ERα selective antagonist in the preparation of a medicament for the treatment of a bone mineral density disorder.

23. The use according to claim 22, in which the ERα antagonist has a binding affinity of ERα of less than 10 nM.

24. The use according to claim 23, in which the ERα antagonist has a binding affinity of 0.0001 to 10 nM for ERα.

25. A pharmaceutical composition suitable for treating or preventing bone mineral density disorders in a mammal, the composition comprising an ERα antagonist or agonist.

26. A pharmaceutical composition according to claim 25, in which the ERα agonist or antagonist has a binding affinity for ERα of less than 10 nM.

27. A pharmaceutical composition according to claim 12, in which the ERα agonist or antagonist has a binding affinity for ERα of 0.0001 to 10 nM.

28. A method of selecting compounds for the regulation of bone mineral density in mammals, the method comprising selecting a compound on the basis of its ability to antagonise agonist-dependent ERα activity.

29. A method according to claim 28, in which compounds are selected for the regulation of adult bone mineral density disorders.

FIG. 1

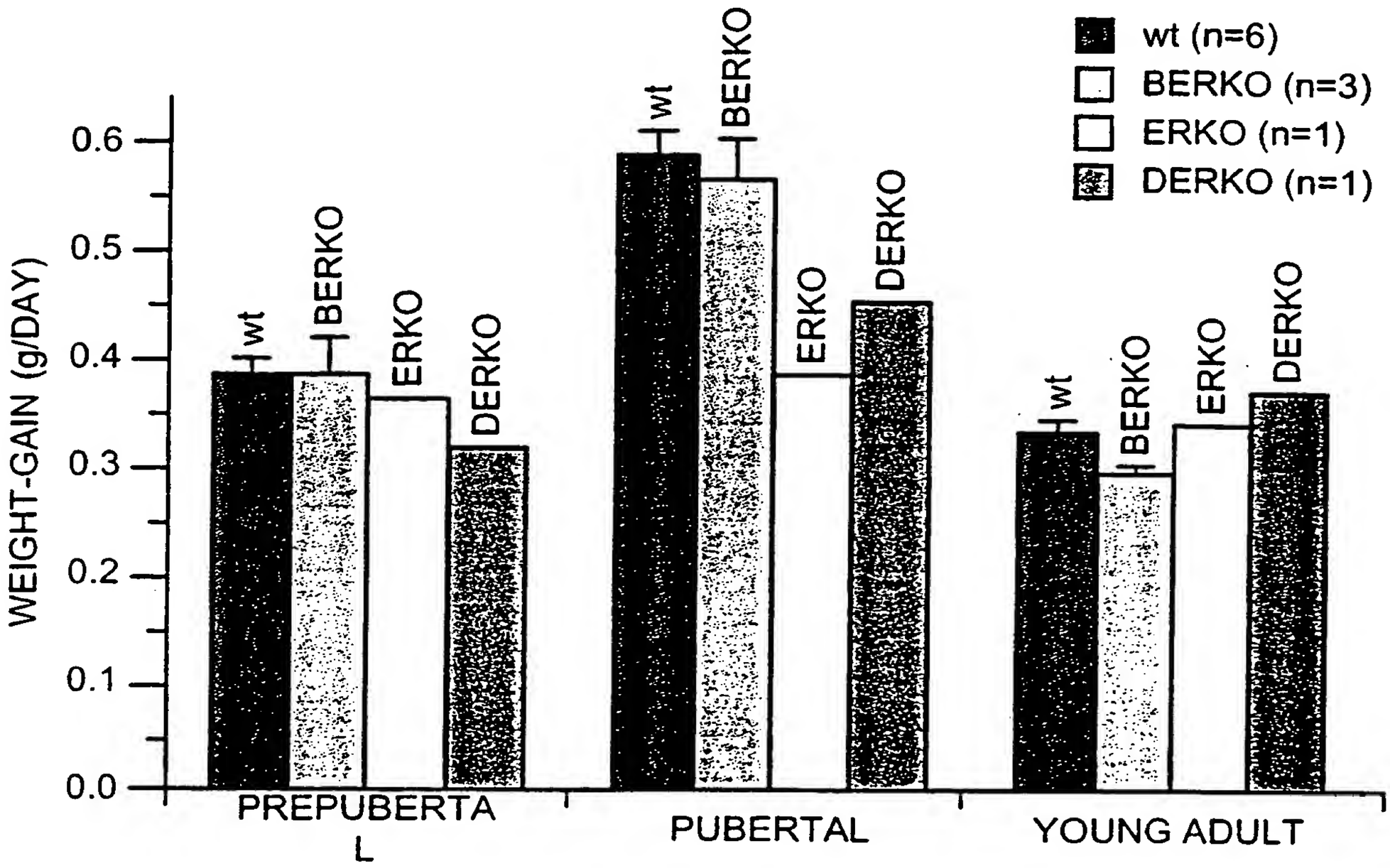
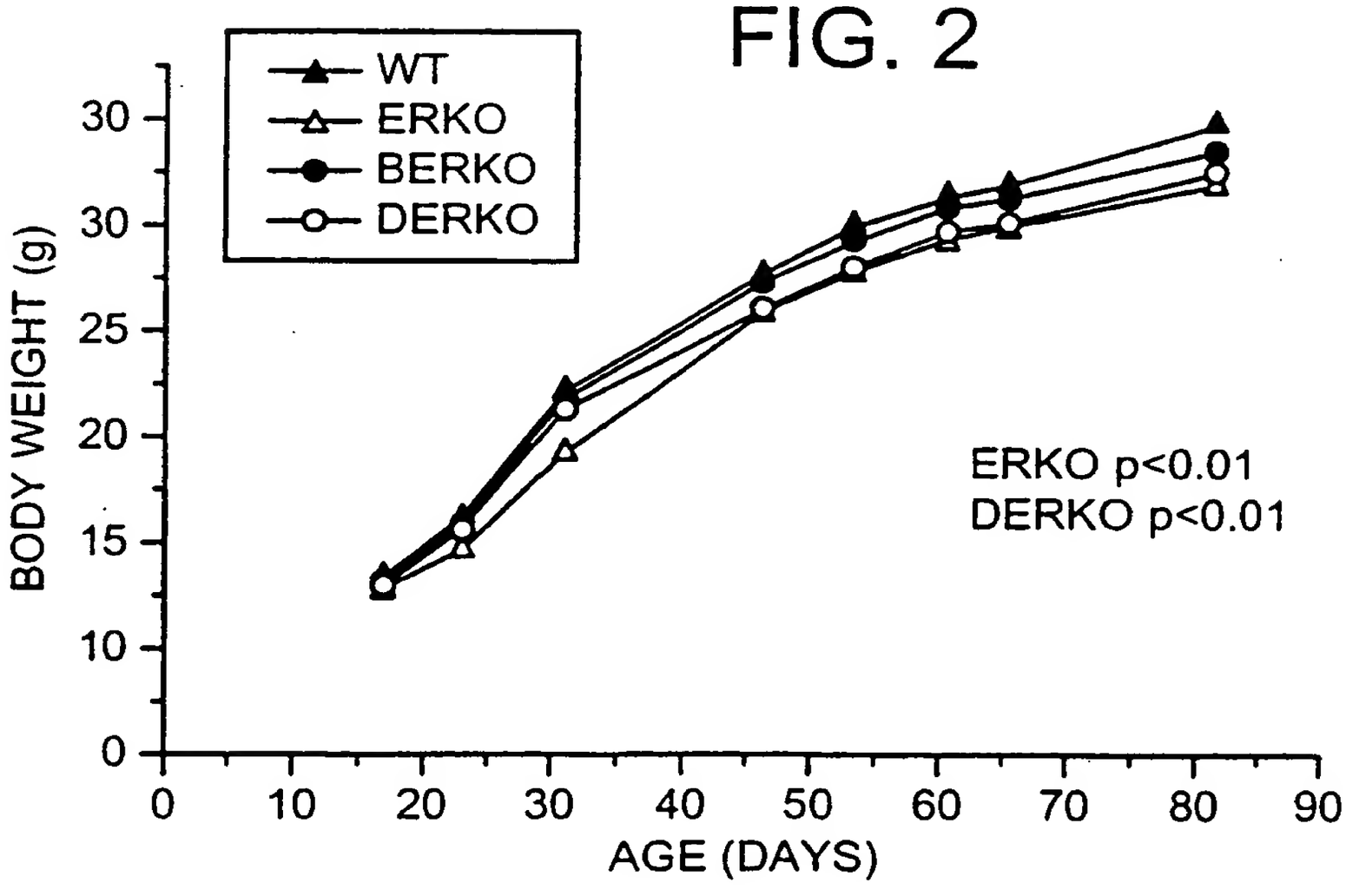

WEIGHT-GAIN MALES (g/DAY)
wt (n=6)
BERKO (n=3)
ERKO (n=1)
DERKO (n=1)
WEIGHT-GAIN (g/DAY)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
wt
BERKO
ERKO
DERKO
PREPUBERTAL
PUBERTAL
YOUNG ADULT

FIG. 2


WT
ERKO
BERKO
DERKO
BODY WEIGHT (g)
0
10
15
20
25
30
35
0
10
20
30
40
50
60
70
80
90
AGE (DAYS)
ERKO p<0.01
DERKO p<0.01

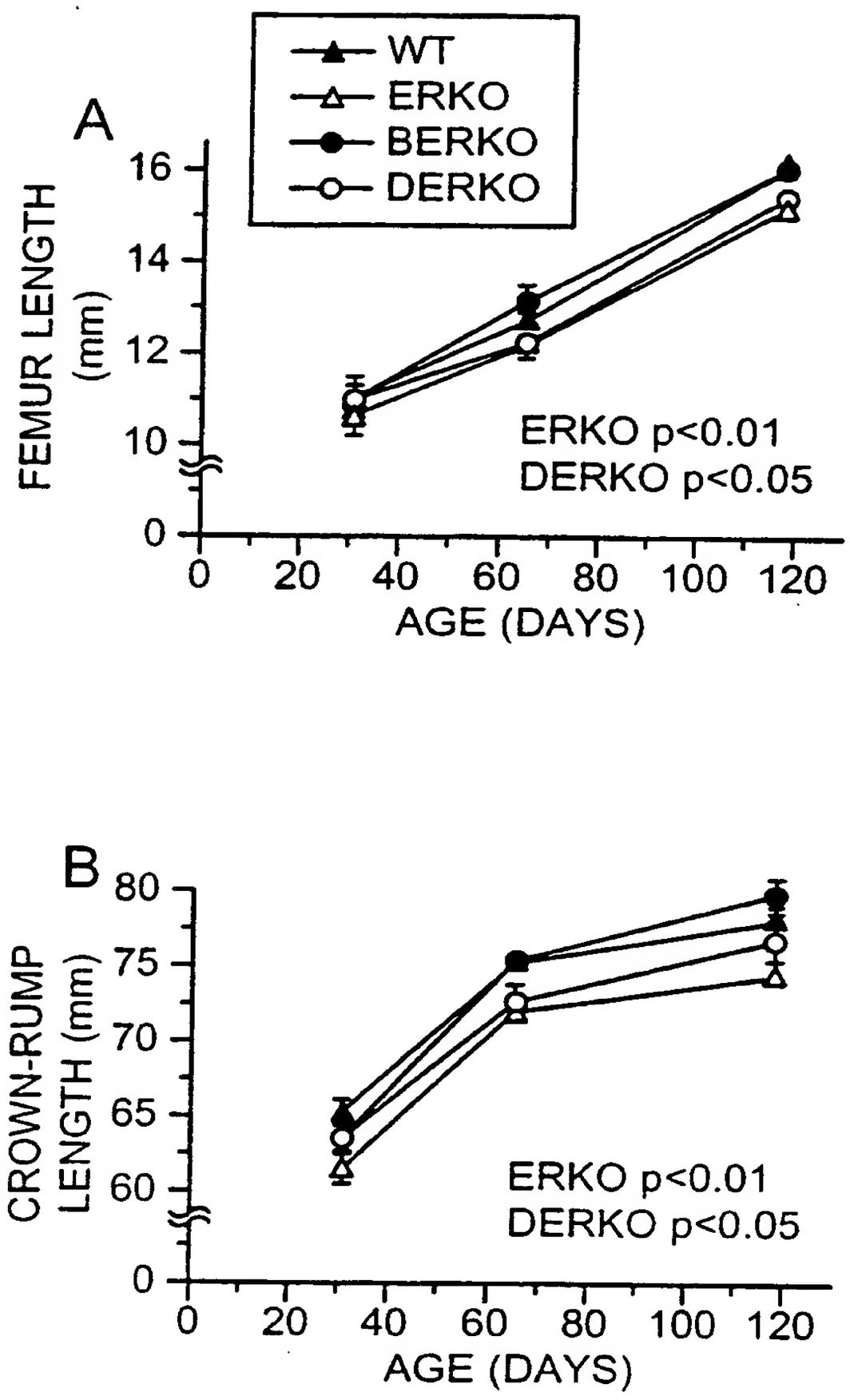

A
WT
ERKO
BERKO
DERKO
FEMUR LENGTH (mm)
16
14
12
10
0
0
20
40
60
80
100
120
AGE (DAYS)
ERKO p<0.01
DERKO p<0.05
B
CROWN-RUMP LENGTH (mm)
80
75
70
65
60
0
0
20
40
60
80
100
120
AGE (DAYS)
ERKO p<0.01
DERKO p<0.05
C
GROWTH PLATE WIDTH (□m)
140
120
100
80
60
40
20
0
WT
ERKO
BERKO
DERKO

FIG. 3

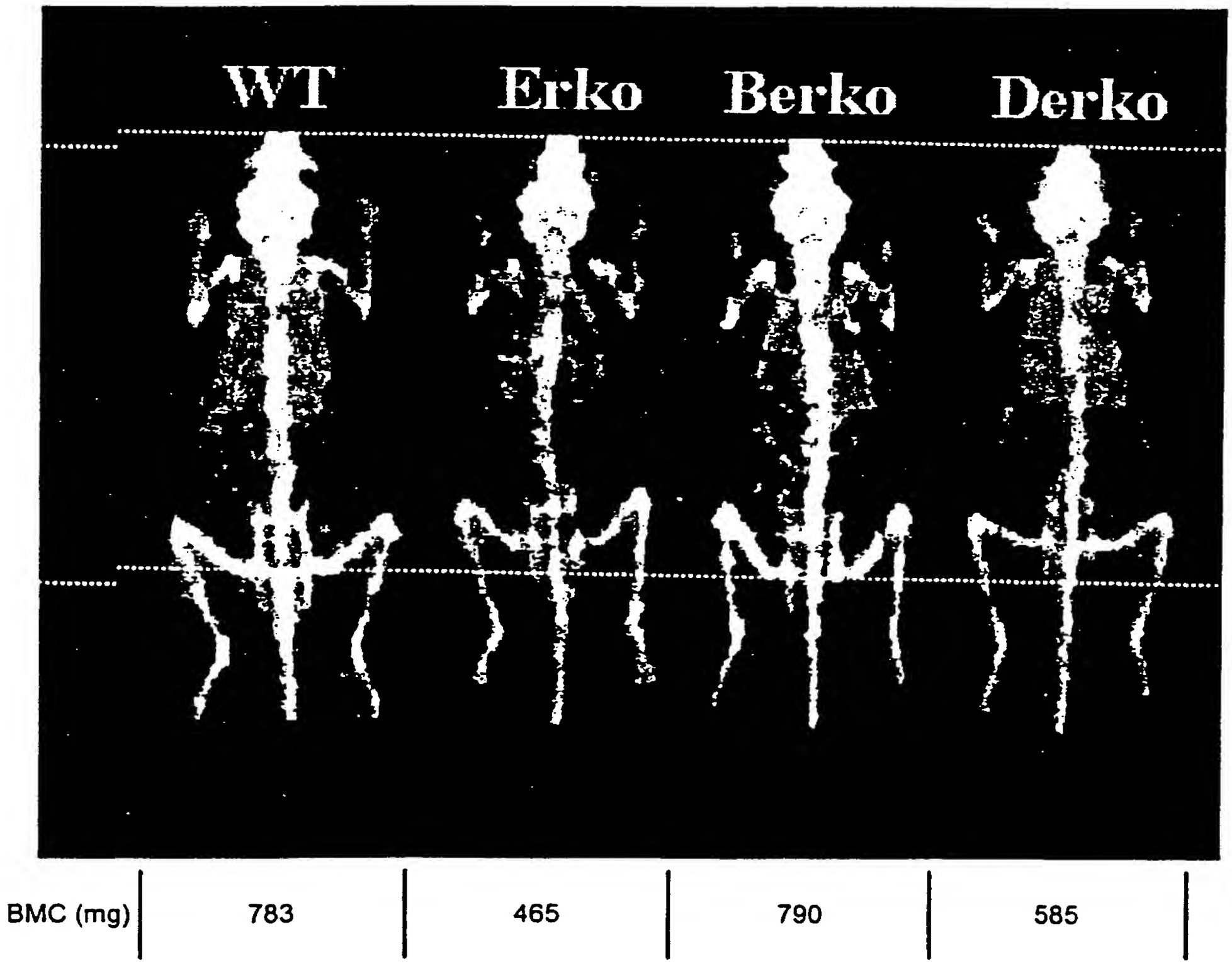
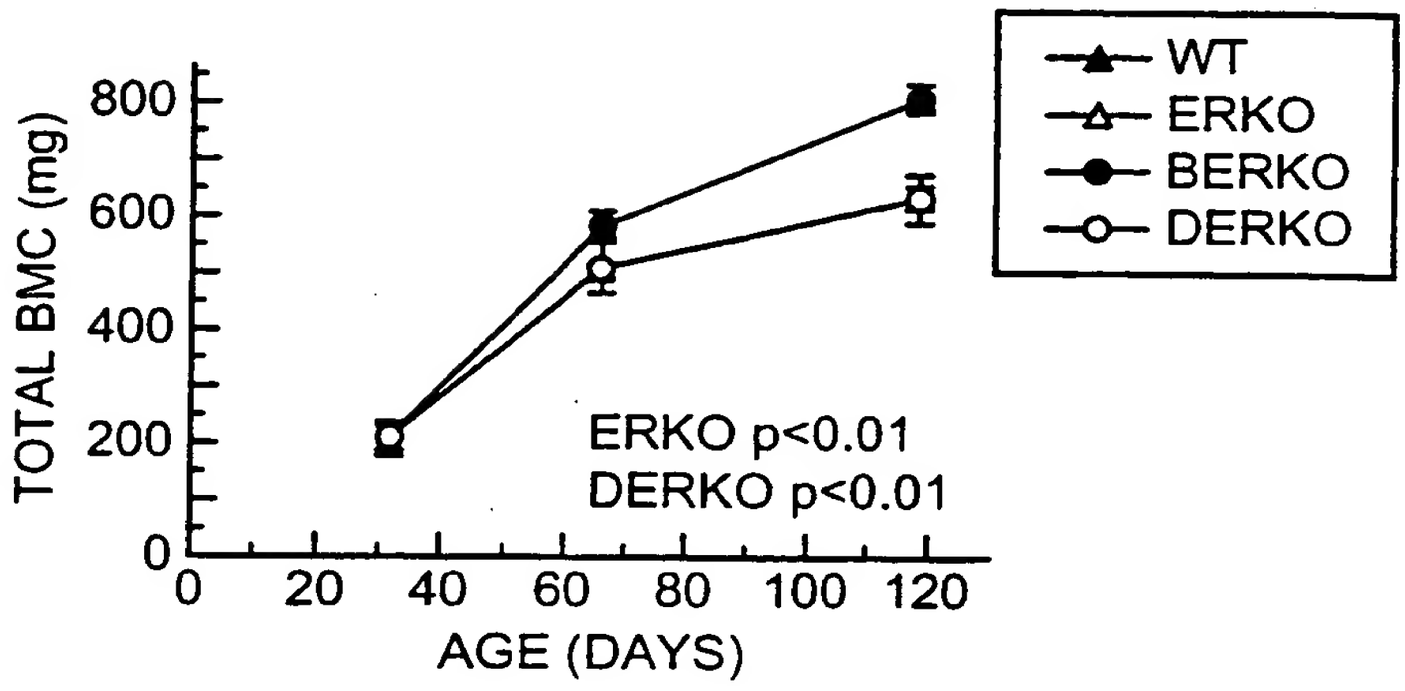
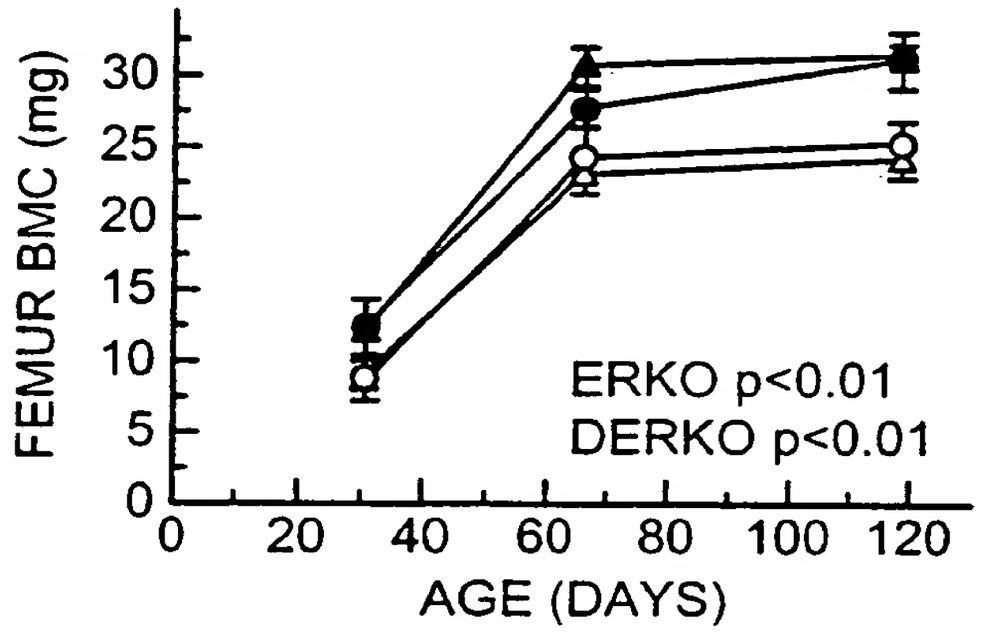
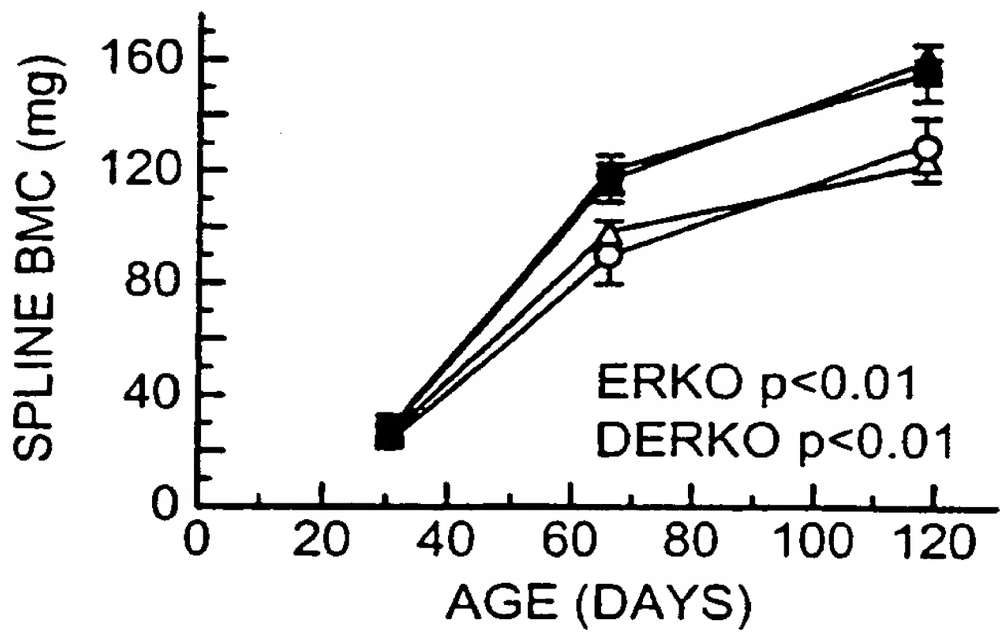


FIG. 4


TOTAL BMC (mg)
800
600
400
200
0
0 20 40 60 80 100 120
AGE (DAYS)
WT
ERKO
BERKO
DERKO
ERKO p<0.01
DERKO p<0.01


FEMUR BMC (mg)
30
25
20
15
10
5
0
0 20 40 60 80 100 120
AGE (DAYS)
ERKO p<0.01
DERKO p<0.01


SPLINE BMC (mg)
160
120
80
40
0
0 20 40 60 80 100 120
AGE (DAYS)
ERKO p<0.01
DERKO p<0.01

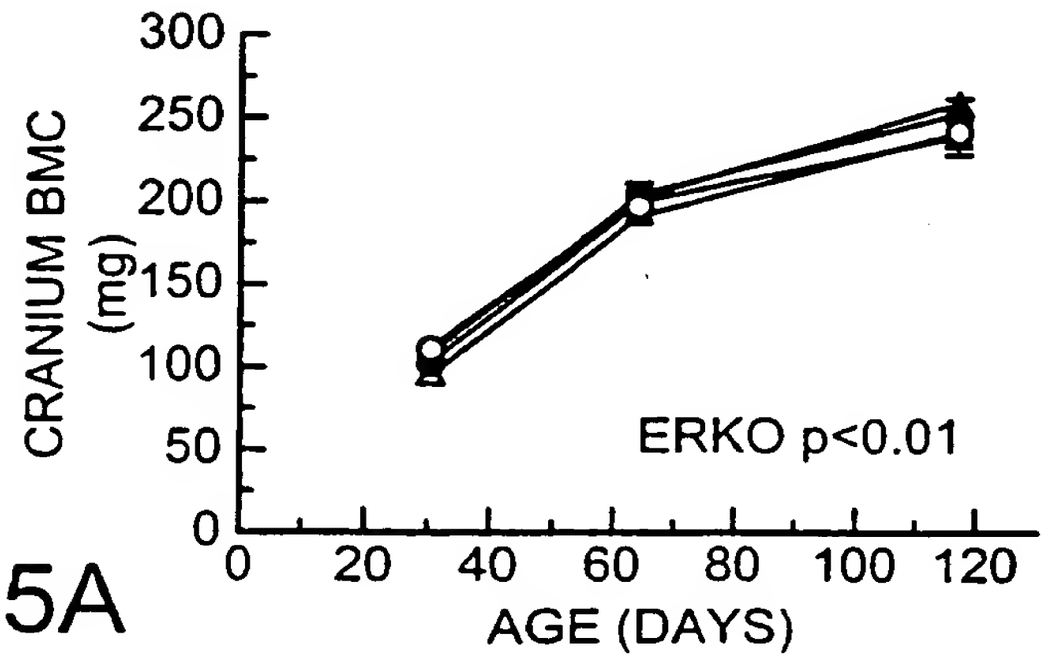
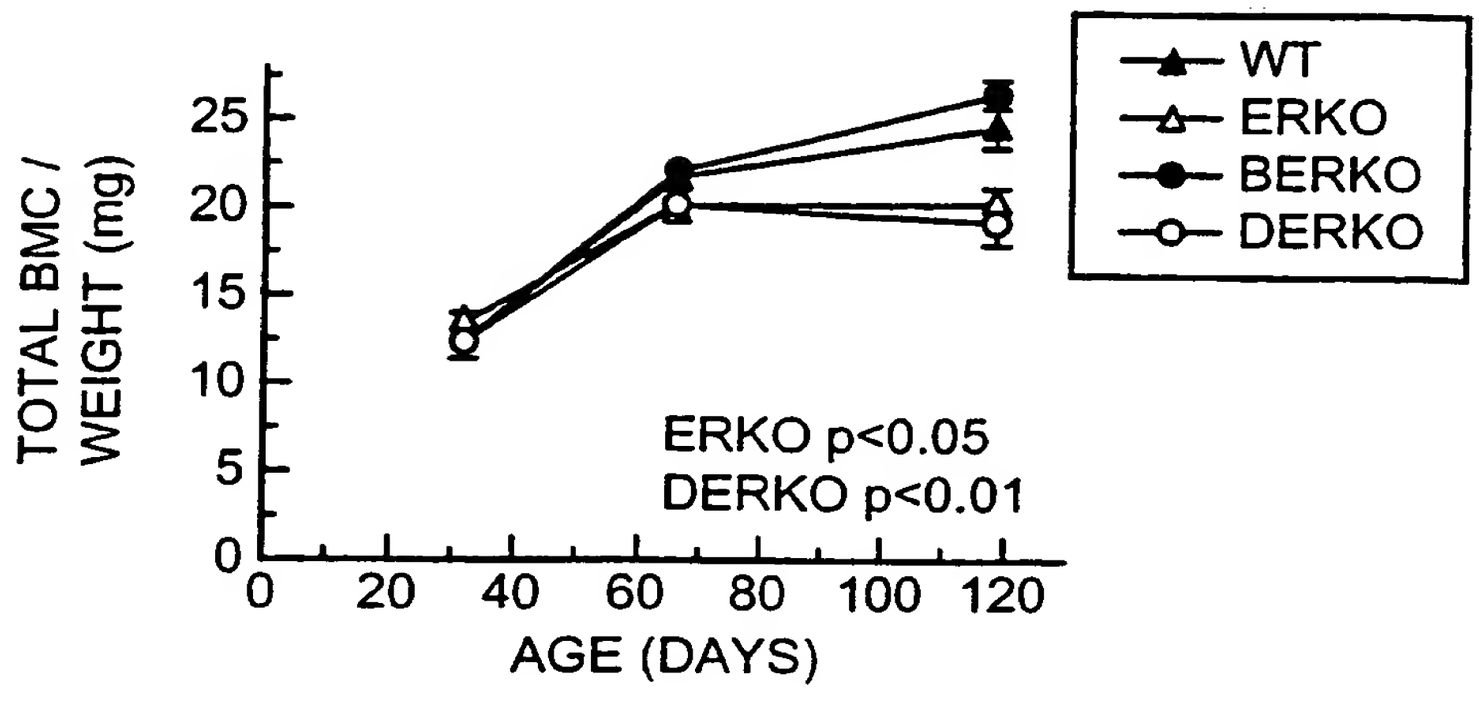
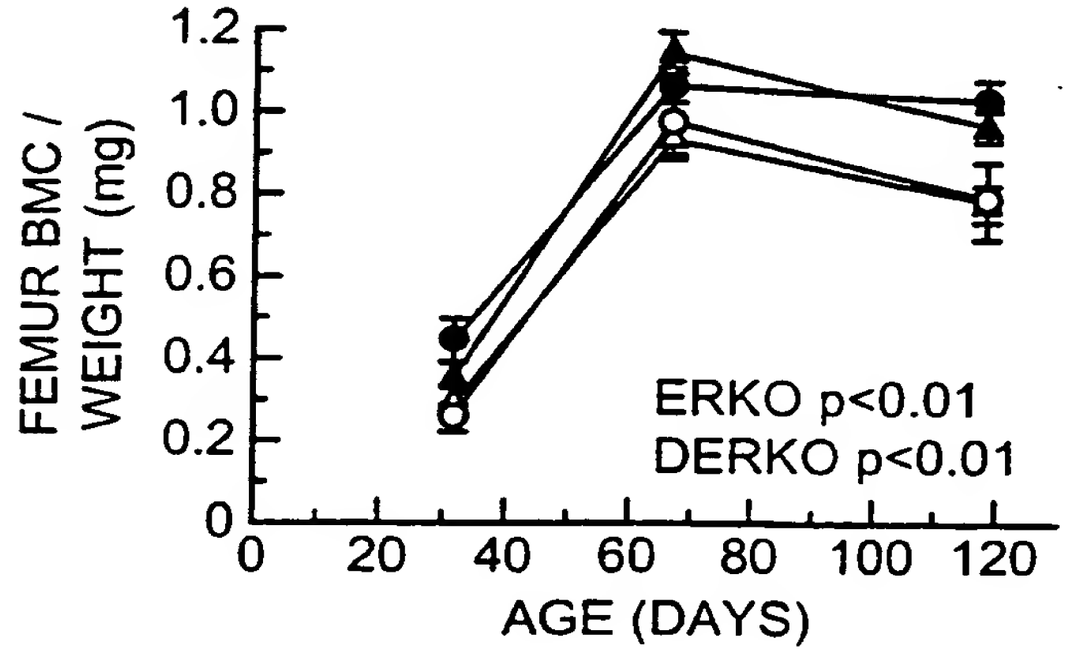
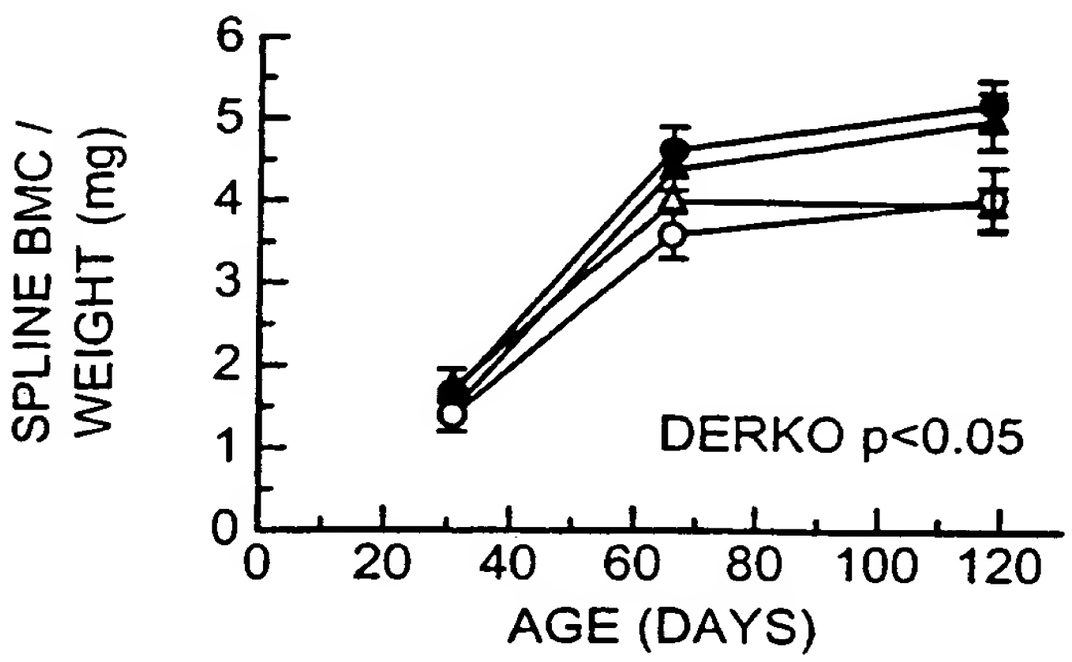

CRANIUM BMC (mg)
300
250
200
150
100
50
0
0 20 40 60 80 100 120
AGE (DAYS)
ERKO p<0.01

FIG. 5A


TOTAL BMC / WEIGHT (mg)
25
20
15
10
5
0
0 20 40 60 80 100 120
AGE (DAYS)
WT
ERKO
BERKO
DERKO
ERKO p<0.05
DERKO p<0.01


FEMUR BMC / WEIGHT (mg)
1.2
1.0
0.8
0.6
0.4
0.2
0
0 20 40 60 80 100 120
AGE (DAYS)
ERKO p<0.01
DERKO p<0.01


SPLINE BMC / WEIGHT (mg)
6
5
4
3
2
1
0
0 20 40 60 80 100 120
AGE (DAYS)
DERKO p<0.05

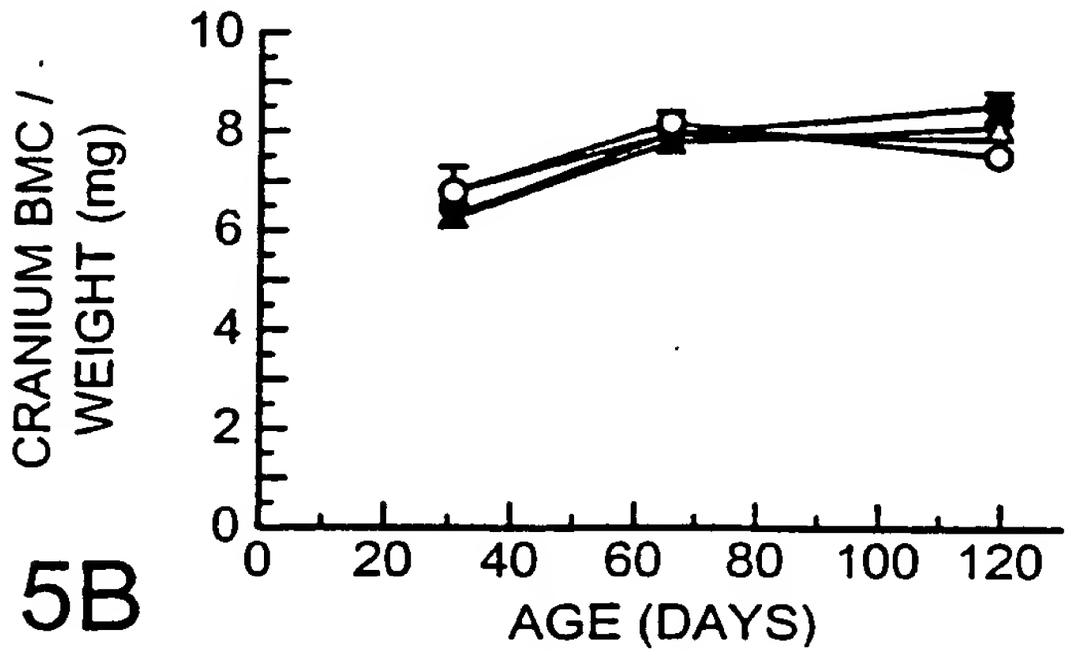

CRANIUM BMC / WEIGHT (mg)
10
8
6
4
2
0
0 20 40 60 80 100 120
AGE (DAYS)

FIG. 5B

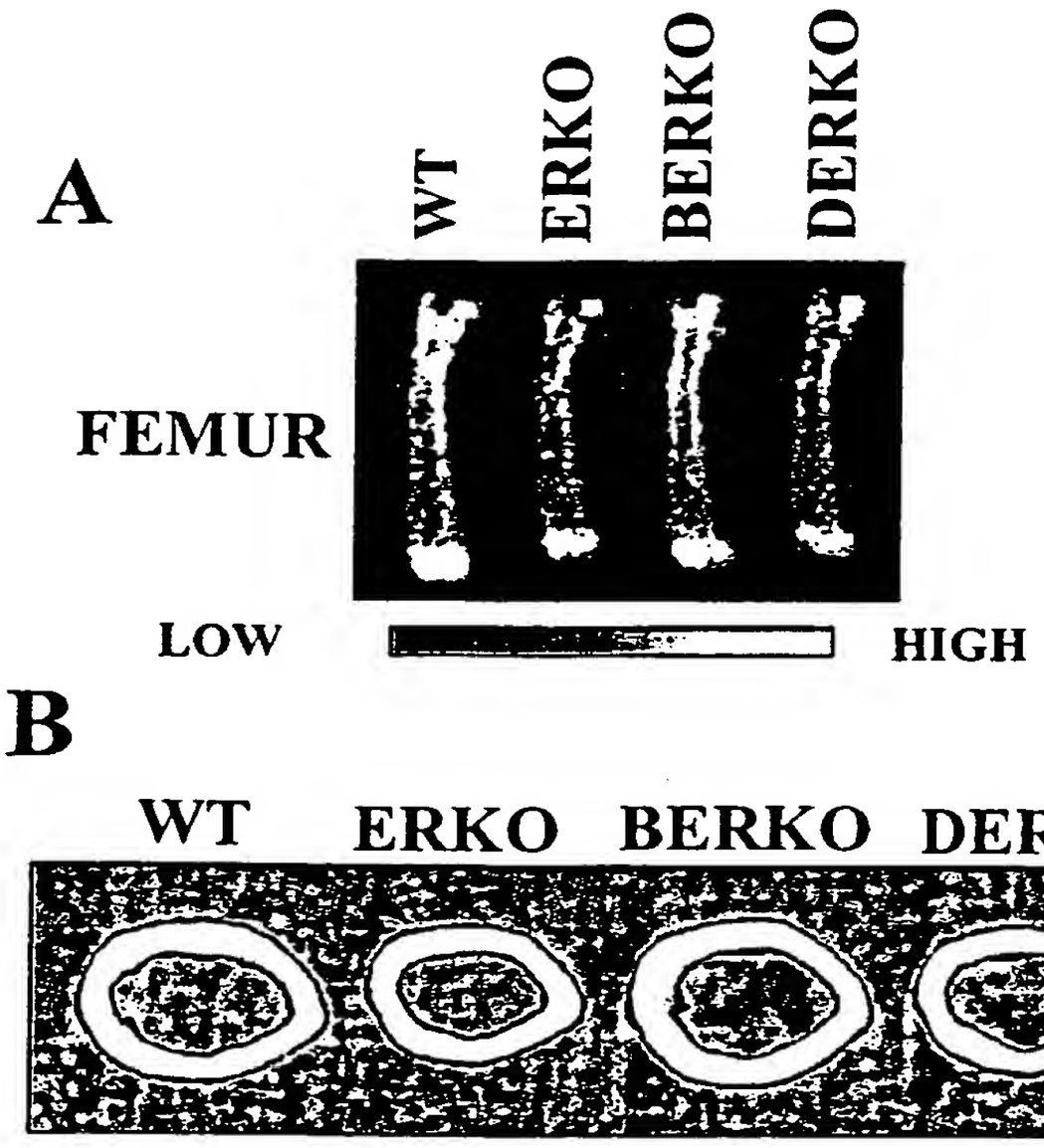
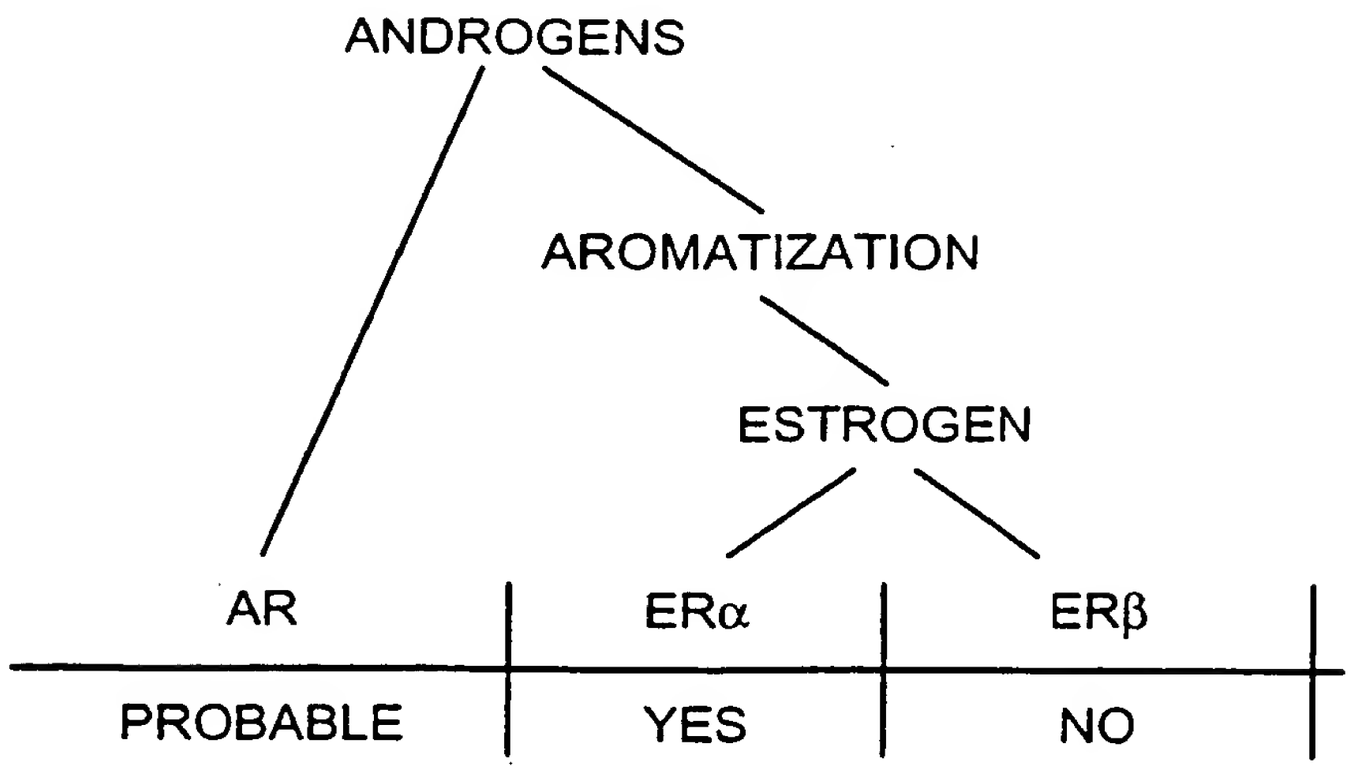


FIG. 6

FIG. 8

EFFECTS OF ANDROGENS IN THE MALE SKELTON



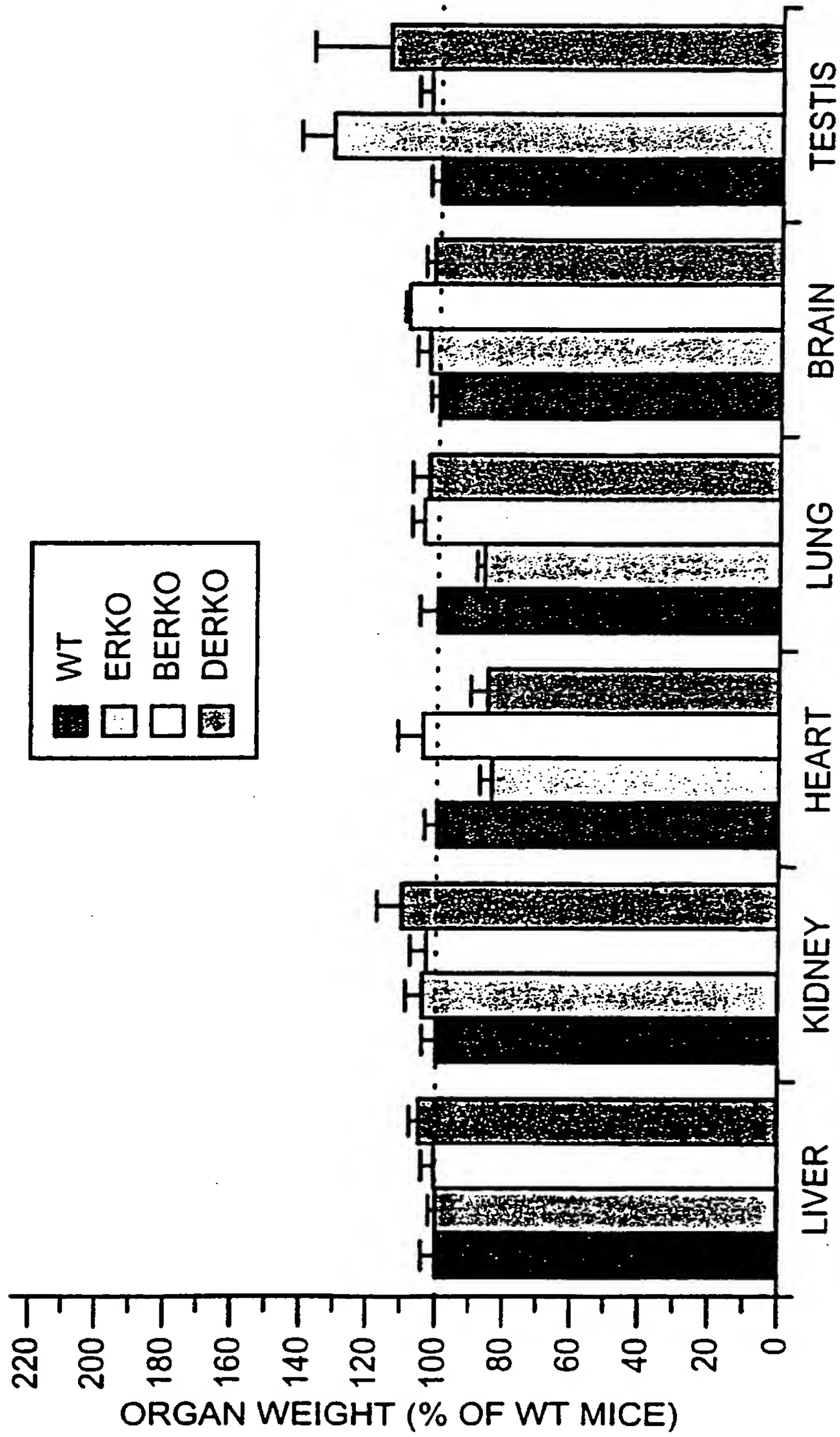

WT
ERKO
BERKO
DERKO
TESTIS
BRAIN
LUNG
HEART
KIDNEY
LIVER
220
200
180
160
140
120
100
80
60
40
20
0
ORGAN WEIGHT (% OF WT MICE)

FIG. 7

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(74) Agent: **DEAN, John, Paul**; Withers & Rogers, Goldings House, 2, Hays Lane, London SE1 2HW (GB).

(81) Designated States *(national)*: AU, CA, JP, US.

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Published:
— *With international search report.*

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12 July 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/76529 A3

(54) Title: USE OF ESTROGEN RECEPTOR AGONISTS OR ANTAGONISTS FOR TREATING GROWTH, BONE DISORDERS

(57) Abstract: Androgens regulate the male skeleton directly via a stimulation of androgen receptors and indirectly via aromatization of androgens into estrogen and thereafter stimulation of estrogen receptors (ER). In order to investigate the relative importance of estrogen receptor subtypes in the regulation of the male skeleton, the skeletal phenotypes of wild type (WT), ERα, Knockout (ERKO), ERβ Knockout (BERKO) and ERα/β Double Knockout (DERKO) mice were compared. ERKO and DERKO had reduced body weight as well as longitudinal bone growth. Furthermore, ERKO and DERKO but not BERKO demonstrated a pronounced decrease in bone mineral content in the long bones and in the axial skeleton. This decrease in BMC was due to cortical ostopenia as a result of decreased radial growth of the bones. Mechanical testing demonstrated that femora from ERKO were weaker as a result of the altered cortical bone dimensions. No significant change in trabecular BMD was seen in any group. ERKO demonstrated decreased serum levels of osteocalcin and IGF-I. Furthermore, serum levels of IGF-I were correlated to most of the skeletal changes seen in DERKO and ERKO. In conclusion , the skeletal phenotypes of DERKO and ERKO are similar and clearly distinguishable from WT and BERKO. Therefore, ERα, but not ERβ, mediates the effect of estrogen in the skeleton of male mice.

Inter nal Application No
PCT/GB 00/02283

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/138 A61P19/00 A61K31/4535 A61K31/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 31640 A (PFIZER) 4 September 1997 (1997-09-04) page 14, line 20-27; claims 1,3,31,32,34,36,66,67,79,80 page 15, line 31 -page 16, line 1 page 16, line 12-23 page 14, line 14 ---	1-4,7,8, 11, 15-18, 21,22,25
X	FRITZ, P. C. ET AL: "Tamoxifen attenuates the effects of exogenous glucocorticoid on bone formation and growth in piglets" ENDOCRINOLOGY (1998), 139(8), 3399-3403 , XP000979648 the whole document --- -/--	1-4,7,8, 11, 15-18, 21,22,25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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°&° document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 January 2001	25/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kanbier, D

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EVANS, GLENDA L. ET AL: "Raloxifene inhibits bone turnover and prevents further cancellous bone loss in adult ovariectomized rats wit established osteopenia" ENDOCRINOLOGY (1996), 137(10), 4139-4144 , XP002088886 the whole document ---	15-17, 21,22,25
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X	WO 98 25623 A (MERCK & CO INC) 18 June 1998 (1998-06-18) page 1, line 6-25; claims 6,9,10,20 ---	1-3,7,8, 11, 15-17, 21,22,25
X	WO 96 02565 A (CELTRIX PHARMA) 1 February 1996 (1996-02-01) claims 1,3,5,9,10,18,19; example 2; tables 5,6 ---	1-3,7,8, 11, 15-17, 21,22,25
X	LABRIE, FERNAND ET AL: "EM-652 (SCH 57068), a third generation SERM acting as pure antiestrogen in the mammary gland and endometrium" J. STEROID BIOCHEM. MOL. BIOL. (1999), 69(1-6), 51-84 , XP000978361 page 72, left-hand column, paragraph 4 -right-hand column, paragraph 1 page 78, left-hand column ---	1-3, 15-17, 21,22,25
X	EP 0 629 697 A (LILLY CO ELI) 21 December 1994 (1994-12-21) claim 5; example XIII ---	14,28,29

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BARKHEM ET AL: "Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists" MOLECULAR PHARMACOLOGY, vol. 54, no. 1, August 1998 (1998-08), pages 105-111, XP000978331 page 105, right-hand column, line 1-3, paragraph 2 page 106, left-hand column, paragraphs 1-4 page 109-111	1-42

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-29 relate to compositions, uses and methods involving compounds defined by reference to desirable characteristics or properties, namely ER-alpha (selective) antagonism and/or agonism. The claims cover all compounds having these characteristics or properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). A compound cannot be sufficiently defined by its mechanism of action and/or its pharmacological profile. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be clear (and/or concise), supported and disclosed. Since there are no examples specifying compounds that will fall within the scope of the present claims, the search has ben restricted to compounds mentioned in the description on page 8, paragraph 3; with due regard to the general idea underlying the application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

...rormation on patent family members

Inter ...nal Application No
PCT/GB 00/02283

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